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Summary

Plant height is an important trait for plant reproductive success. Plant height is often under pollinator-mediated selection, and has been shown to be correlated with various other traits. However, few studies have examined the evolutionary trajectory of plant height under selection and the pleiotropic effects of plant height evolution. We conducted a bi-directional artificial selection experiment on plant height with fast cycling *Brassica rapa* plants to estimate its heritability and genetic correlations and to reveal evolutionary responses to artificial selection on height and various correlated traits. With the divergent lines obtained through artificial selection, we subsequently conducted pollinator-choice assays and investigated resource limitation of fruit production. We found that plant height variation is strongly genetically controlled (with a realized heritability of 41% to 59%). Thus, plant height can evolve rapidly under phenotypic selection. In addition, we found remarkable pleiotropic effects in phenology, morphology, floral scent, color, nectar, and leaf glucosinolates. Most traits were increased in tall line plants, but flower size, UV reflection,

and glucosinolates were decreased, indicating potential trade-offs. Pollinators preferred plants of the tall selection lines over the short selection lines in both greenhouse settings with bumblebees and field experiment with natural pollinators. We did not detect any differences in resource limitation between plants of the different selection lines. Overall, our study predicts that increased height should evolve under positive pollinator-mediated directional selection with potential trade-offs in floral signals and herbivore defense.

Introduction

Most angiosperms interact with both mutualistic pollinators and antagonistic visitors via various plant traits. Plant or inflorescence height is commonly measured in selection studies and has often been shown to be under positive pollinator-mediated selection (Donnelly *et al.* 1998, Gómez 2003, Irwin 2006, Ehrlén *et al.* 2012, Ågren *et al.* 2013, Fenster *et al.* 2015, Gervasi and Schiestl *in prep.*), though some studies have shown negative (Cariveau *et al.* 2004) or a lack of pollinator-mediated selection (Parachnowitsch and Kessler 2010, Sletvold *et al.* 2015). Plant height is also important for seed dispersal, as higher plants are predicted to disperse their seed further (Thomson *et al.* 2011). In addition, antagonists such as herbivores (Gómez 2003, Ågren, et al. 2013), nectar robbers (Irwin 2006), or seed/fruit predators (Cariveau, et al. 2004, Kolb and Ehrlén 2010, Ehrlén, et al. 2012) can impose negative selection on plant height. Selection on plant height, however can only contribute to plant trait evolution if there is a genetic background of the trait (Endler 1986).

Genetic variance and covariance are the two main components that determine and modify plant trait evolution after selection (Endler 1986, Falconer 1989, Roff 1997). On the one hand, heritability, the genetic basis of phenotypic variation, is a pre-requisite for a trait's adaptive evolution (Falconer 1989, Roff 1997). The heritability (h^2) of plant height has been

repeatedly studied over years (*e.g.* Billington et al. 1988, Andersson 1991, Charlesworth and Mayer 1995, Andersson 1996, Bennington and McGraw 1996, Andersson 1997, Bonnin et al. 1997; see review in Geber and Griffen 2003). The majority of these studies revealed plant height to be significantly heritable with h^2 varying from 10% to 75%. Additionally, studies of quantitative genetics in various species have identified various quantitative genes with several alleles for plant height (Beavis *et al.* 1991, Song *et al.* 1995, Kato *et al.* 1999, Yu *et al.* 2003, Li *et al.* 2013, Zhang *et al.* 2015), providing genetic pre-requisite for plant height evolution. On the other hand, genetic covariance, or the standardized genetic correlation with other plant traits will potentially affect plant trait evolution via indirect responses to selection (Campbell 1996, Mitchell *et al.* 1998, Caruso 2004). Plant height has been documented to correlate with other traits such as flower number (Andersson 1996, Gómez 2003), flower size (Andersson 1996), and plant phenology (Charlesworth and Mayer 1995, Andersson 1996, Bonnin, et al. 1997). Such trait covariance can be caused by genetic pleiotropy (the same gene with different phenotypic effects) or linkage disequilibrium (different genes with different effects but linked) (Falconer 1989, Roff 1997). Particularly negative correlations, known as trade-offs, can also result from resource limitation (Agrawal *et al.* 2010). Overall, ample genetic background (genetic variance and covariance) has been demonstrated for plant height, providing the potential of plant height evolution under certain selection. However, studies that examined how plant height evolution under selection leads to concerted changes in traits and pollinator responses are not yet available.

Experimental evolution and artificial selection are vital tools to follow the evolutionary trajectory of traits under controlled conditions. In this study, we used fast cycling *Brassica rapa* to carry out bi-directional artificial selection on plant height for three successive generations. Fast cycling *B. rapa* plants originate from fast flowering individuals from accessions of the USDA (United States Department of Agriculture) plant germplasm system

(Williams and Hill 1986a). These plants were subsequently selected for short generation time, rapid seed maturation, absence of seed dormancy, small plant size, and high female fertility, yet retain high genetic variability (Miller and Schemske 1990, Ågren and Schemske 1992, Zu *et al.* 2016). We obtained estimates for plant height heritability and its genetic/phenotypic correlations, and also observed indirect responses of other correlated traits. In addition to commonly measured morphological and phenological traits, we included several plant traits that are not commonly quantified, such as floral scent, floral color, nectar sugar compounds, and leaf glucosinolates. We also assessed the consequences of plant height evolution on attractiveness for pollinators, and assortative pollinator visitation. Pollinators often visit flowers/plants of the same type consecutively, the so-called floral constancy, which can lead to assortative pollen flow and reproductive isolation (Kay and Sargent 2009). Specifically, the objectives of this study were to: (1) detect whether and how plant height, as well as other plant traits, respond to artificial selection; (2) estimate plant height heritability and its genetic/phenotypic correlations with other traits; (3) test whether pollinators have preferences for plants of the different selection lines; and (4) examine whether the ability to produce fruits (resource limitation) differs in different plant lines after artificial selection.

Results

Direct and indirect responses to artificial selection on plant height

In the artificial selection experiment, directional selection for both tall and short plants resulted in strong changes in plant height (Fig. 1). All three lines (tall, short, control) started from the same parental population with a mean (± SE) plant height of 26.0 ± 0.45 cm. In the 3rd offspring generation, the height of the plants had changed to 41.9 ± 0.65 cm, 21.0 ± 0.57 cm, and 32.0 ± 0.76 cm in the tall, short, and control lines, respectively ($P < 0.001$, Fig. 1).

The realized heritability of plant height was 0.406 ± 0.015 (P = 0.001) estimated from the tall line, and 0.588 ± 0.091 (P = 0.023) estimated from the short line.

In addition to significant changes of plant height after three generations of artificial selection, we found pleiotropic responses in diverse traits including flower size, phenology, scent, color, nectar and leaf glucosinolates (Fig. 2). The taller plants had more but smaller flowers (Table 2; Fig. 2b), larger flower distance (*i.e.* distance between two adjacent flowers), longer flower petioles, and took longer time to flower (Fig. 2b). The taller plants also produced more nectar (Fig. 2b) with higher amounts of all the three measured sugar compounds (Fig. 2c); all three sugar compounds and nectar volume were strongly correlated with one another (the correlation coefficient ranging from 0.748 to 0.961, all $P < 0.001$). Consequently, the nectar concentration did not differ among the lines ($P > 0.1$, one-way ANOVA).

Various floral volatiles (except benzaldehyde and four nitrogen containing benzenoid/phenylpropanoid compounds) were produced in significantly higher amounts in the tall line plants than in the short line plants ($P < 0.05$, Fig. 2a). Petals of plants in the tall line reflected less UV than those of the short and control plants ($P < 0.001$, Fig. 2e). The total amount of leaf glucosinolates and several individual glucosinolates decreased in tall line plants and increased in short line plants ($P < 0.05$, Fig. 2d). The overall floral phenotype differed among all three lines (linear discriminant analysis, $P < 0.001$ for both axes, Fig. 2f). The average variance of all these traits was similar among all three lines ($P = 0.846$, one-way ANOVA).

Heritabilities and genetic correlations of plant traits from pedigree data

Narrow-sense heritability based on an 'animal model' fitted with MCMCglmm showed that all the traits were significantly heritable. Plant height showed a heritability of 58.2% (Table 1), which was similar to the realized heritability. Flower size (petal width, petal length,

flower diameter) exhibited an average heritability of 51.3% (Table 1). Heritability of log-transformed flower nectar amount was 27.3%, of sugar compounds was between 9.6% to 23.1% (Table 1). In addition, heritability of floral volatile organic compounds (VOCs) (log-transformed) was ranging from 0.2% to 64.4%, with an average of 40.7% (Table 1), which is much larger than what we found in a previous study of the same species (Zu, et al. 2016) probably due to here applied log transformation of data (raw scent data distributions were highly skewed towards zero).

Among all the possible pairs of the 17 floral traits that we measured in all the generations, the average phenotypic correlation coefficient was 0.136 and the average genetic correlation coefficient was 0.180, with the highest values among different flower size measurements, and among benzenoid/phenylpropanoid floral VOCs (Fig. 3). Genetic and phenotypic correlation coefficients were strongly correlated ($r = 0.912$, $P < 0.001$, Mantel test). Seventy-three (out of 136) pairs of phenotypic correlation were significant, whereas 29 (out of 136) pairs of genetic correlation were significant (Fig. 3).

Pollinator choice

In the dual-choice experiment, bumblebees chose to land on tall-line plants three times more often than on short-line plants ($P < 0.001$, Fig. 4). In the floral constancy assay, bumblebees paid their 1st visit more often to tall than to short plants ($P < 0.001$, Fig. 4), but did not discriminate between them during the 2nd and 3rd visits ($P > 0.05$, Fig. 4). The resulting pollinator constancy index was 0.55, which means that pollinators switched randomly between tall and short line plants. In fact, our observations suggested that bumblebees, after their first visit, usually visited the neighboring plant regardless of its size, a pattern predicted by optimal foraging. The duration that the bumblebees spent on each plant was not significantly correlated with plant height or available flower numbers ($P > 0.1$, general linear

model). This may, however, have been influenced by our attempt to reduce the differences of open flower number between high and low line plants for our experimental setting (see in experimental procedures section).

In the field experiment with natural pollinators, plants from tall line received significantly more pollinator visitations than plants from the short line. Syrphid flies (*Eristalis tenax*) were the most abundant pollinator group and accounted for most of the differences (Fig. 4). Consequently, tall line plants had significantly higher proportional fruit set (80.5% ± 0.023) than short line plants (68.0% ± 0.025, $P < 0.001$, One-way ANOVA).

Resource limitation of fruit/seed production

The tall line plants produced significantly more flowers and fruits than the short line plants in the F3 generation (Table 2). However, all the three lines were able to develop around 40% to 46% of pollinated flowers into fruits, and therefore there were no significant differences in resource limitation for fruit production among all three lines (Table 2). All lines produced a similar number of seeds per fruit (Table 2). However, fruits and seeds of the tall line plants were significantly heavier than those of short line plants (Table 2).

Discussion

Pollinator-mediated selection for taller plants is common in nature (Donnelly, et al. 1998, Gómez 2003, Irwin 2006, Ehrlén, et al. 2012, Ågren, et al. 2013, Fenster, et al. 2015, Gervasi and Schiestl *in prep.*). However, little is known about the consequences of such selection, and the pleiotropic effects of an evolutionary increase in plant height. Artificial selection experiments are a direct way to observe evolutionary responses to selection (Falconer 1989; Roff 1997). Our experiment showed that plant height of fast cycling *Brassica rapa* responded

fast, predictably and continuously to artificial selection in both up- and down-direction. These results suggest that plant height harbors substantial genetic variance and is inherited as a quantitative trait in *Brassica rapa*. Plant height increase also resulted in correlated responses of non-target traits due to genetic correlations between the selected and non-selected traits (Falconer 1989, Roff 1997, Conner 2003). These striking pleiotropic effects include some novel findings of antagonistic pleiotropies in leaf glucosinolates and UV reflection and mutualistic pleiotropies in floral scent and nectar.

Mechanisms of genetic correlations

Genetic correlations are caused by either gene pleiotropy or linkage disequilibrium (LD) (Falconer 1989; Roff 1997). It is likely that the changes of flower distance and petiole length were mainly due to pleiotropic effects of height genes such as the *Le* gene (Mendel's stem length gene) that controls the level of gibberellin acid and affects cell elongation and cell division (Lester *et al.* 1997, Martin *et al.* 1997). In contrast, the genetic correlation between plant height and flowering time has been shown in several studies to be caused by LD of long-term co-selection of distinct genes (Lin *et al.* 1995, Bezant *et al.* 1996, Ming *et al.* 2002, Zhang, et al. 2015). Indeed, the fast cycling *Brassica rapa* has been selected artificially both early flowering and short plant (Williams and Hill 1986b), which is likely to have mediated the observed genetic correlation between these two traits.

The correlations between plant height and biochemical traits such as nectar production or floral scent can be caused by linkage disequilibrium stemming from historical selection or by potential "up-regulation pleiotropies". Those two mechanisms are not necessarily mutually exclusive. On the one hand, correlations between plant height, nectar and volatiles can form honest signals that pollinators may select for (Raguso 2004, Wright and Schiestl 2009, Knauer and Schiestl 2015). Therefore, such selection can lead to LD among these traits. On

the other hand, a recent transcriptome study has shown that selection on a single scent compound (phenylacetaldehyde) leads to higher level of many other VOCs, due to an up-regulation of genes involved in the production of shared precursor compounds , as well as ribosomal protein genes and thus the gene-translational machinery (Cai *et al.* 2016). Thus, if upregulation of genes involved in plant height also increases the level of ribosomal protein production and/or related metabolism, such up-regulation pleiotropy could cause augmentation of various biochemical traits when plant height is selected for. To discriminate LD from pleiotropy, subsequent studies exerting large-scale random pollination could be conducted. Genetic correlations caused by linkage disequilibrium can be easily disrupted through random mating for a few generations (Falconer 1989; Roff 1997) whereas pleiotropy cannot. In addition, transcriptomics studies on floral traits can potentially reveal mechanisms of genetic correlations on gene-expression level (Cai, et al. 2016).

Negative correlations and allocation trade-offs

Negative correlations usually reflect trade-offs when resources are a limiting factor (Van Noordwijk and de Jong 1986, Worley and Barrett 2001, Agrawal, et al. 2010). In our study, despite the dominating positive correlations, we also found evidence for antagonistic pleiotropies for flower size, leaf glucosinolates and UV reflectance. Glucosinolates are the main defense compounds in Brassicaceae (Textor and Gershenzon 2009), and have been shown to be costly to produce (Mauricio 1998, Züst *et al.* 2011, Bekaert *et al.* 2012). Their reduced production in tall plants could be a physiological trade-off which contributes to a potential ecological trade-off between pollinator attraction and defense as shown in other studies (Strauss *et al.* 1999, Agrawal 2011). Although we show here that tall line plants are more attractive to pollinators, it remains to be investigated whether short plants are better defended through the production of more glucosinolates. In addition to glucosinolates, an antagonistic pleiotropic effect was apparent for UV reflectance and petal size. UV reflectance

has been shown to correlate with petal size in a study across 300 species (Guldberg and Atsatt 1975). If the same pattern applies for a within species level, the smaller petal size in our high line plants provides an explanation for the reduced UV reflectance. In contrast to these antagonistic pleiotropies, we did not find any trade-offs between plant height and floral scent or nectar production. Although little is known about the costs of these traits, both have been suggested to be costly at least in some instances (Vogel 1962, Southwick 1984, Pyke 1991, Gershenzon 1994). Our study suggests that such costs are negligible in our model plant, as tall plants with their increased scent and nectar production showed no sign of reduced resource limitation in fruit production, but on the contrary produced even more and heavier seeds than short line plants.

Predicting plant trait evolution

To understand and predict trait evolution in nature, we need estimations of selection gradients and the genetic architecture (genetic variance/covariance matrices) of the traits (Falconer 1989, Roff 1997, Harder and Johnson 2009). Our pollinator behavior experiments, as well as numerous earlier selection studies have shown that pollinators are able to distinguish tall versus short plants and thus impose positive selection on plant height. In nature, selection often only acts on a few traits (Johnson and Steiner 1997, Alexandersson and Johnson 2002, Gomez *et al.* 2008, Sletvold and Ågren 2010, Sletvold *et al.* 2010, Schiestl *et al.* 2011, Parachnowitsch *et al.* 2012, Gross *et al.* 2016). Our dataset predicts that even with selection acting only on plant height, *B. rapa* plants should become more fragrant, more rewarding, but have smaller flowers with less UV reflection and less defensive glucosinolates. In an earlier study, Zu et al (2015) have shown that the evolution of individual fragrance components is also strongly linked by pleiotropy. Obviously, a more conclusive understanding of selection, including antagonist-mediated selection (Ågren, et al. 2013), are needed to attain a more realistic model of trait evolution in nature.

Experimental procedures

Plant species

Brassica rapa L. (syn. *B. campestris*: Brassicaceae) is a self-incompatible annual or biannual plant with a generalized pollination system (including bumblebee *Bombus terrestris*, honey bees *Apis mellifera*, hoverflies like *Episyrphus balteatus*, and butterflies like *Pieris brassicae*, or *P. rapae* being some of its pollinators). In our experiment, rapid cycling *B. rapa* from the Wisconsin Fast Plants^{TM} Program (Carolina Biological Supply Company, Burlington, NC, USA) was used. This rapid cycling line needs only *ca.* 35 days to complete a life cycle and maintains sufficient genetic variability for selection experiments (Miller and Schemske 1990, Ågren and Schemske 1992, Zu, et al. 2016).

We sowed out seeds of rapid cycling *B. rapa* plants in a phytotron at the Botanical Garden of the University of Zürich, with 24h fluorescent light per day, 22°C, 60% relative humidity; we watered twice a day (at 08:00 and 18:00). After one week, we pricked each seedling into an individual pot (7cm*7cm*8cm) with standardized soil (Humuswerke Gebr. Patzer GmbH & Co.KG, Sinntal, Germany) and subsequently kept them in the same phytotron until fruit set and seed maturation.

Artificial selection experiment

We carried out a bi-directional artificial selection experiment on plant height to produce "tall", "short", and control plants. At the start of the experiment, we grew 150 rapid cycling *Brassica rapa* plants as the initial parental population (P). On the 23rd day after sowing we measured plant height (from the soil surface to the top of the inflorescence) for selection. Day 23 is about one week after flowering; height on the 23rd day was strongly correlated with final plant height ($r = 0.81$, $P < 0.001$). We first randomly chose 10 plants to initiate the control line group; then we selected the 10 tallest plants and 10 shortest plants as the "tall" and

"short" line groups respectively. We then randomly hand-pollinated four flowers on each of the chosen plants within their own line to obtain the seeds for the next generation. After fruit maturation we sowed out six to seven randomly-selected seeds from each of the 10 chosen plants in all the three lines so that each line would have *ca.* 50 effective descendants in the first offspring generation (F1). We then repeated the same height measurement, artificial selection and hand pollination procedures until the third offspring generation (F3). A total of 580 plants were thus sampled and analyzed in this study.

Plant and floral traits measurement

For all generations, besides plant height we measured flower diameter, and petal length and width (using a digital caliper) of two flowers per plant. Nectar from at least 4 freshly opened flowers (open for one to two days) per plant was collected with 1µl microcapillaries (Brand GmbH & Co.KG, Wertheim, Germany). To analyze sugar compounds (glucose, fructose, sucrose) in nectar, the collected nectar from microcapillaries was derivatized and analyzed using gas chromatography (GC-MSD) (details in supporting information materials and methods).

Floral volatile organic compounds (VOCs) from the whole inflorescence with at least 4 freshly opened flowers were collected using a push-pull system as described in Zu *et al.* (2016). The entire inflorescence of each individual was enclosed in a cylindrical glass vessel with silanized (deactivated) surfaces (Sigmacote, Sigma, MO, USA). The vessel containing the inflorescence was closed at the bottom with two Teflon plates with an opening in the middle for the stem. Two glass ports at the upper and the middle part of the vessel could be closed with an open screw cap fitted with a penetrable PTFE septum. Through one port a filter with activated charcoal (SKC Inc. Eighty Four, PA, USA) was introduced, connected to an air pump pushing purified air at a flow rate of 100ml min^{-1} into the glass vessel. Through

the other port a glass tube filled with adsorbent (35mg Tenax TA 60/80, Supelco, Bellefonte, PA, USA) was introduced, connected to a vacuum pump for pulling out air containing inflorescence volatiles at the same flow rate. We collected scent for three hours from each plant in the phytotron. Because no significant daily dynamics of scent emission were found in a pilot experiment, we collected scent during one of the following time periods: 07:00 – 10:00, 12:00 – 15:00, and 17:00 – 20:00, depending on the available number of qualified plants. We collected at least one air control sample from an empty glass cylinder in each collection period. To analyze floral VOCs, we used a gas chromatograph with a mass-selective detector (GC-MSD) fitted with a thermal desorption system (Gerstel TDS/TDU, Mülheim an der Ruhr, Germany). Each sample was loaded and injected into the GC (Agilent 6890N, Agilent Technologies, Palo Alto, CA, USA) using a Gerstel MultiPurpose Sampler MPS. The details for thermal desorption program were identical to Zu *et al.* (2016) (see supporting information materials and methods). An Agilent 5975 Series MSD mass spectrometer was used for compound identification and quantification. To identify volatile compounds, mass spectra obtained from the samples were compared with those of a reference collection (the National Institute of Standards and Technology (NIST) mass spectral library). Subsequently, retention times and mass spectra of all compounds included in the quantitative analyses were compared to those of synthetic reference standards. Compound quantification was carried out with the ChemStation Enhanced Data Analysis program (Version E.01.00). For all compounds included in the study, synthetic standards were analyzed in three different amounts using GC-MSD (1, 10 and 100 ng). By selecting specific target ions for each compound, calibration curves were established using the ChemStation program. Peak areas of target ions were subsequently used for quantifying the amounts of volatiles in the samples.

All compounds were standardized in units of ng flower^{-1} liter^{-1} of sampled air. VOCs that were either relatively scarce (present in less than 20% of all the samples) or found in small

amounts (comparable to the amounts in air controls) were excluded from the analyses. In the end, a total of 12 floral VOCs were quantified in this study. One floral VOC that was included in Zu *et al.* (2016), (*Z*)-3-hexen-1-ol, could not be detected in this study due to the shortening of the column of GC-MSD machine. This compound, however, was strongly correlated with another floral VOC (Z)-3-hexenol acetate (Zu *et al.*, 2016).

Furthermore, from a subset of the plants in the last offspring generation, we also recorded the number of days from sowing out to flowering, and we measured flower petiole length (from the 5^{th} and the 6^{th} freshly opened flower), flower distance (distance between two adjacent flowers: we measured distance between the 5^{th} and the 6^{th}, and between the 6^{th} and the 7^{th} freshly opened flower), flower color of two fresh petals per plant (see supporting information materials and methods for measurement details), as well as leaf glucosinolates, the typical defense secondary metabolites of the Brassicaceae (for extraction and analysis methods, see supporting information materials and methods). Glucosinolates in leaf and flower were previously found to be strongly correlated (Schiestl 2014).

Pollinator behavior experiment

To test whether pollinators have a preference for plants from one of the selection lines, we performed dual-choice and floral constancy experiments in greenhouse, as well as pollinator behavior experiment in the field.

For the two greenhouse experiments, we used the bumblebee pollinator, *Bombus terrestris* (Apidae) (obtained from Andermatt Biocontrol, Andermatt, Switzerland). All bumblebees were previously exposed to a mixed array of F3 plants from all three lines in order to allow them to learn the plant and flower signals.

In the dual-choice experiment, we placed one tall plant against one short plant with a similar numbers of open flowers in a flight cage (76cm*76cm*115cm, length*width*height), at a distance of 12 cm apart from each other. One bumblebee per time was released into the cage to make its choice. As soon as the bee landed on one plant, it was caught and immediately released to another cage with plants to prevent further usage of the same bee. At the end of the day, these bees were released back into their hive. Six bumblebees were assayed one by one for each pair of plants. The position of the paired plants was always swapped after each pollinator landing. In total, 15 pairs of high and low plants with a total of 90 bumblebee visits were used in this experiment.

In the flower constancy bioassay, we tested whether plant size differences can lead to assortative visitation by bumblebees and thus to floral isolation between plants of different height. We set up an array (six plants per row, five rows) with 30 plants (15 high and 15 low) in the center of a flight cage (250cm*180cm*120cm, length*width*height). The plants were positioned with a distance of 12 cm between each other. Each plant had both tall and short plants randomly positioned as neighbors to allow the bee pollinators' free choice. We try to match tall and short plants with similar number of open flowers ($P = 0.977$, one-way ANOVA). Plant height and the number of open flowers were recorded prior to the experiment. We released one bumblebee at a time and allowed it to visit three plants in succession. For all the three visits, we recorded the plant IDs and the duration of the visit. Thereafter, the bee was caught and immediately released to another cage with plants to prevent further usage. At the end of the day, these bees were released back into their hive. Ten bumblebees were used one by one in this experiment, in total resulting in 30 visits for the 30 plants. Two blocks of the floral constancy bioassay were conducted.

To test whether natural pollinators in the field have any preference for the divergent lines, we set up an array of 88 plants (44 plants from tall line and 44 from short line) in an open field at the Botanical Gardens of the University of Zürich. These plants were grown in the greenhouse for 22 days (with at least 6 freshly opened flowers) before being transferred into the field. Plants were placed with a distance of 30 cm between each other to form a plant block of eight rows and eleven columns. Each plant had both tall and short plants randomly positioned as neighbors to allow the bee pollinators' free choice. We conducted pollinator observation in every ten minutes of the first one hour after setting up the plant array. There were several pollinator species, including honeybee (*Apis mellifera*), syrphid flies (*Eristalis tenax*), and some other undefined syrphid fly species. These pollinators were grouped into three categories (*i.e.* honeybees, *Eristalis tenax*, and other syrphid flies). During pollinator observation, we went through each column of the block to record presence/absence of pollinator, pollinator categories and abundances for each plant. In total six rounds of pollinator observations were conducted. The plants were then left in the field for another two hours to allow for more pollinator visitation. We then transferred these plants back to the greenhouse to set fruits. Total number of open flowers were counted prior to the experiment and total number of developing fruits were counted one week after the experiment.

Resource limitation experiment

To detect different degrees of resource limitation in F3 plants of the three lines, we randomly selected 17 plants of each line and out-crossed all the flowers of each plant over the entire flowering period. The out-crossed pollen donors were taken from two or more plants in different lines to reduce the chance of inbreeding and incompatibility. We recorded total flower number and number of produced fruits for each plant. After fruit maturation, we randomly chose two fruits from each plant and measured fruit weight, number of seeds per fruit and seed weight.

Data analyses

To calculate realized heritability on plant height, we used the breeder's equation:

$h^2 = R/S$,

with heritability (h^2) as the slope (with associated standard error SE) of the linear regression of the cumulative response to selection (R) on the strength of selection (*i.e.* the cumulative selection differential S) (Falconer 1989, Hill and Caballero 1992, Roff 1997). We forced the regression through the origin (0, 0) because all lines came from the same initial population. The selection differential was calculated as the difference between the mean plant height of the selected plants and all measured plants in the same generation. The response to selection was measured as the difference between the mean plant height of the selected plants in generation i and all measured plants in generation i -1. To control for random variation between generations, we subtracted the mean value of the control line from the mean value in the tall and short lines in each generation to standardize the calculations.

To test whether there were significant differences between tall, short, and control lines for all the measured traits in the last generation, we conducted separate one-way ANOVAs for all traits. *Post hoc* tests (Tukey honestly significant difference (HSD)), with the significance level set to 0.05 were applied to examine which specific lines differed from each other. For flower color, we first reduced the variables into principal components (PCs) that explain 95% of the total variance, and then performed a discriminant function analysis (DFA) to examine overall differences among the lines in the last generation. To assess the differences of the overall phenotypes among lines in the last generation, we performed a LDA (linear

discriminant analysis) for most of the measured traits (some traits i.e. color, nectar and leaf glucosinolates were excluded due to strong reduction of the sample size).

We were also able to calculate narrow-sense heritability, phenotypic and genetic correlations of plant height and other measured traits by pooling all the generations and using the pedigree relationship of all these plants. A Bayesian framework fitted with generalized linear mixed model (*MCMCglmm* package version 2.22.1 (Hadfield 2010)) was adopted to calculate heritability and phenotypic and genetic correlations. Generation and line were taken as fixed effects; dam and sire were entered as random effects. We used weakly informative inverse-Wishart prior with limit variance of one and covariance of zero (i.e. V = diag(n), where "n" is the number of traits used in analysis), and low degree of belief (0.002). Posterior distributions were robust to several prior settings (e.g. V = diag(n)*0.1, V=diag(n)*10). We used iterations of 1200000, burn-in 200000 and thinning 500 to ensure convergence and low autocorrelation among thinned samples. Nectar and flower VOCs were log transformed (by ln(x +1), x is raw value) prior to analysis in order to get normalized distributions.

From the model, we obtained estimates of additive genetic variance (V_A), among-dam variance (V_D), among-sire variance (V_S) and residual variance (V_R). Narrow-sense heritability (h^2) was calculated as V_A/V_P (Falconer 1989), where V_P is the phenotypic variance calculated by ($V_A + V_D + V_S + V_R$). Heritability was significant if 95% HPD (highest probability density) interval did not overlap zero. Narrow-sense heritability was calculated from univariate analysis by taking each trait per time. Genetic and phenotypic correlations were calculated from standardized genetic variance (V_A) and phenotypic variance (V_P) respectively. Correlation significances were given according to 95% HPD intervals. Genetic and phenotypic correlations were estimated from multivariate analysis of sets of traits, in which days to flowering and sugar compounds in nectar were excluded due to

largely missing measurement for F2 generations. We used posterior mode (the most likely value) instead of posterior mean (the average value from posterior distribution) for heritability and correlation calculation.

To compare the similarity between phenotypic and genetic correlation matrices, a Mantel test (Mantel 1967, Cheverud 1988, Roff 1995) was applied, permutations was set to 10000 times.

To test for pollinator preference for tall and short line plants, we used a binomial test with the data from the dual-choice experiment and with the choices of each of the three visits from the bioassays. To test for any relationship between visit duration and plant height or available flower number, we used a general linear model. Based on the number of switches of bumblebees between tall and short line plants, we pooled the data from the two blocks of bioassays and calculated floral constancy (FC) index:

$$FC = 1 - \frac{number\ of\ interspecific\ visits}{number\ of\ total\ visits},$$

The interspecific visits here mean visits switching between different lines. The FC index ranges from 0 to 1, where 1 represents complete constancy to one plant type, whereas 0.5 refers to random visitation, and 0 refers to completely disassortative visits.

In the field pollinator behavior experiment, the numbers of pollinator visits from honeybees, *Eristalis tenax*, other syrphid flies and total number of visits were summed for each plant from the six rounds of pollinator observations. ANOVAs were used to test the differences of pollinator visits between tall and short plants. Proportional fruit set, the proportion of flowers that turned into fruit after pollination was taken as a measurement of plant fitness and were compared between tall and short plants by ANOVA.

To estimate resource limitation, we again used proportional fruit set. A value of 1 indicates no resource limitation, when all fertilized flowers became fruits. Lower values would indicate stronger resource limitation. Additionally, some fruit and seed properties (*i.e.* number, weight) as well as the total flower number of plants in the resource limitation experiment were compared in separate one-way ANOVAs for all the lines. *Post hoc* Tukey HSD tests with the significance level set to 0.05 were again used to examine differences between specific lines. All these statistical analyses were performed with R version 3.2.0.

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Supporting Information Legends

Supporting Materials and Methods. Details of some methods parts, *i.e.* nectar sugar compound analysis, thermal desorption settings for floral scent analysis, floral color measurement, and glucosinolate analysis can be found in the Supporting information part of this article.

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Tables

Table 1. Narrow-sense heritability and additive genetic variance (posterior mode and 95% HPD) of the 21 measured traits in *Brassica rapa*. Nectar related traits and floral scent data were log transformed by ln(x +1), where x is raw value.

			heritability			additive genetic variance		
Trait	**Abbr.**	**Category**	h^2	**95% HPD**		V_A	**95% HPD**	
Days to flower	DF	phenology	0.4017	(0.1676,	0.5745)	0.2911	(0.1064,	0.4706)
Height	H	morphology	0.5819	(0.4616,	0.7060)	15.8173	(11.2780,	20.7378)
Petal width	PW	morphology	0.4551	(0.2520,	0.5802)	0.1635	(0.0849,	0.2413)
Petal length	PL	morphology	0.5459	(0.4158,	0.6958)	0.1674	(0.1152,	0.2340)
Flower diameter	FD	morphology	0.5387	(0.3603,	0.6697)	0.8126	(0.5279,	1.1522)
Nectar	Nect	nectar	0.2731	(0.1381,	0.4167)	0.4208	(0.2279,	0.6253)
Fructose	Fruc	nectar	0.2306	(0.0432,	0.4730)	1.1921	(0.3361,	2.3230)
Glucose	Gluc	nectar	0.2010	(0.0380,	0.4992)	1.9694	(0.3063,	2.6372)
Sucrose	Suc	nectar	0.0957	(0.0247,	0.2054)	0.2930	(0.1287,	0.4522)

Methyl salicylate	MeS	scent; BNZ	0.6443	(0.4789,	0.7804)	0.5686	(0.3795,	0.7765)
Methyl benzoate	MeB	scent; BNZ	0.5300	(0.3565,	0.6388)	0.3460	(0.2270,	0.4884)
Methyl anthranilate	MeA	scent; BNZ, N-	0.4250	(0.2818,	0.6207)	0.5005	(0.2815,	0.8107)
2-Amino benzaldehyde	ABen	scent; BNZ, N-	0.3203	(0.1509,	0.5018)	0.4940	(0.1984,	0.8166)
Indole	Ind	scent; BNZ, N-	0.4507	(0.2458,	0.6478)	0.3539	(0.1841,	0.6219)
Benzylnitrile	BenN	scent; BNZ, N-	0.4723	(0.2927,	0.6298)	0.2423	(0.1363,	0.3525)
Phenylacetaldehyde	PAA	scent; BNZ	0.6236	(0.4325,	0.7682)	1.0995	(0.6942,	1.5251)
Phenylethyl alcohol	PhA	scent; BNZ	0.5132	(0.3176,	0.6426)	0.3586	(0.2242,	0.5592)
Benzaldehyde	Ben	scent; BNZ	0.5010	(0.3164,	0.6263)	0.2027	(0.1305,	0.3179)
α-Farnesene	FAR	scent; TP	0.1878	(0.0612,	0.3505)	0.0857	(0.0225,	0.1809)
(Z)-3-Hexenyl acetate	ZHA	scent; FAD	0.2221	(0.0046,	0.4571)	0.1266	(0.0013,	0.2839)
1-Butene-4-isothiocyanate	ITC	scent; N-; S-	0.0020	(0.0002,	0.2194)	0.0037	(0.0003,	0.3361)

BNZ, benzenoids/phenylpropanoids; TP, terpene; FAD, fatty acid derivative; N-, nitrogen-containing compound; S-, sulfur-containing compound.

Table 2. Mean (± SE) of plant reproductive traits for the three lines after three generations of artificial selection in *Brassica rapa* (statistically significant values in bold).

	Control line (N = 16)	**Tall line (N=18)**	**Short line (N=18)**	**F**	**P**
Total no. flower	129.250 ± 12.554	151.353 ± 17.328	93.294 ± 8.467	4.933	**0.011**
Total no. fruits	53.250 ± 4.865	57.176 ± 5.448	40.647 ± 3.227	3.58	**0.036**
Proportion of fruit set	0.421 ± 0.028	0.401 ± 0.023	0.464 ± 0.044	0.954	0.393
Weight of fruits (mg)	78.716 ± 3.705	81.986 ± 2.901	50.289 ± 2.042	37.015	**<0.001**
Seeds per fruit	23.375 ± 1.223	22.917 ± 0.675	20.750 ± 1.214	1.769	0.181
Weight of seeds (mg)	2.296 ± 0.066	2.306 ± 0.087	1.680 ± 0.715	22.675	**<0.001**

Figure legends

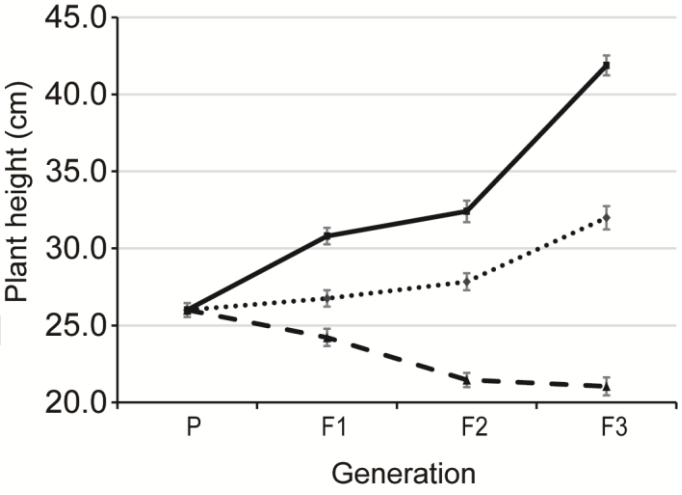
Figure 1. Responses to artificial selection on plant height (mean ± SE, cm) in *Brassica rapa* over three generations (F1, F2, F3, starting from parental generation P). The tall selection line is solid, the short line dashed, and the control line dotted.

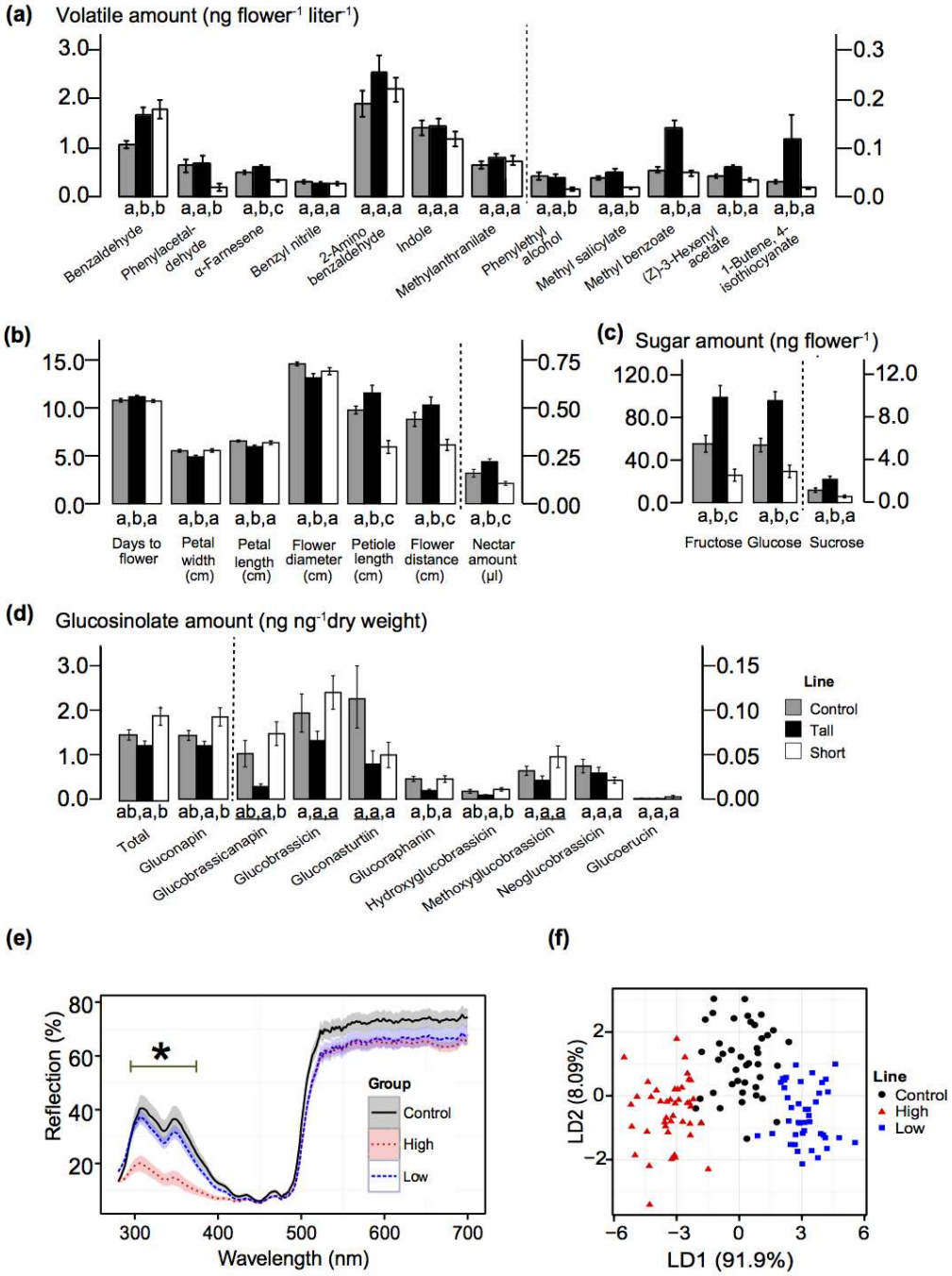
Figure 2. Values (mean ± SE) of various plant traits in the three lines of *Brassica rapa* after three generations of artificial selection on plant height: (a) floral volatiles, (b) other floral traits, (c) nectar sugar compounds, (d) leaf glucosinolates, (e) floral color, (f) overall floral phenotype (linear discriminant analysis). Different letters below bars indicate significant differences between lines (Tukey's tests, $P < 0.05$); letters with underline in (2d) indicate marginal significances ($0.05 < P < 0.1$). The values left of the dashed line follow the y-axis on the left, those to the right side of the dashed line follow the y-axis on the right. The star in (2e) indicates that the corresponding UV wavelength range differs significantly among the

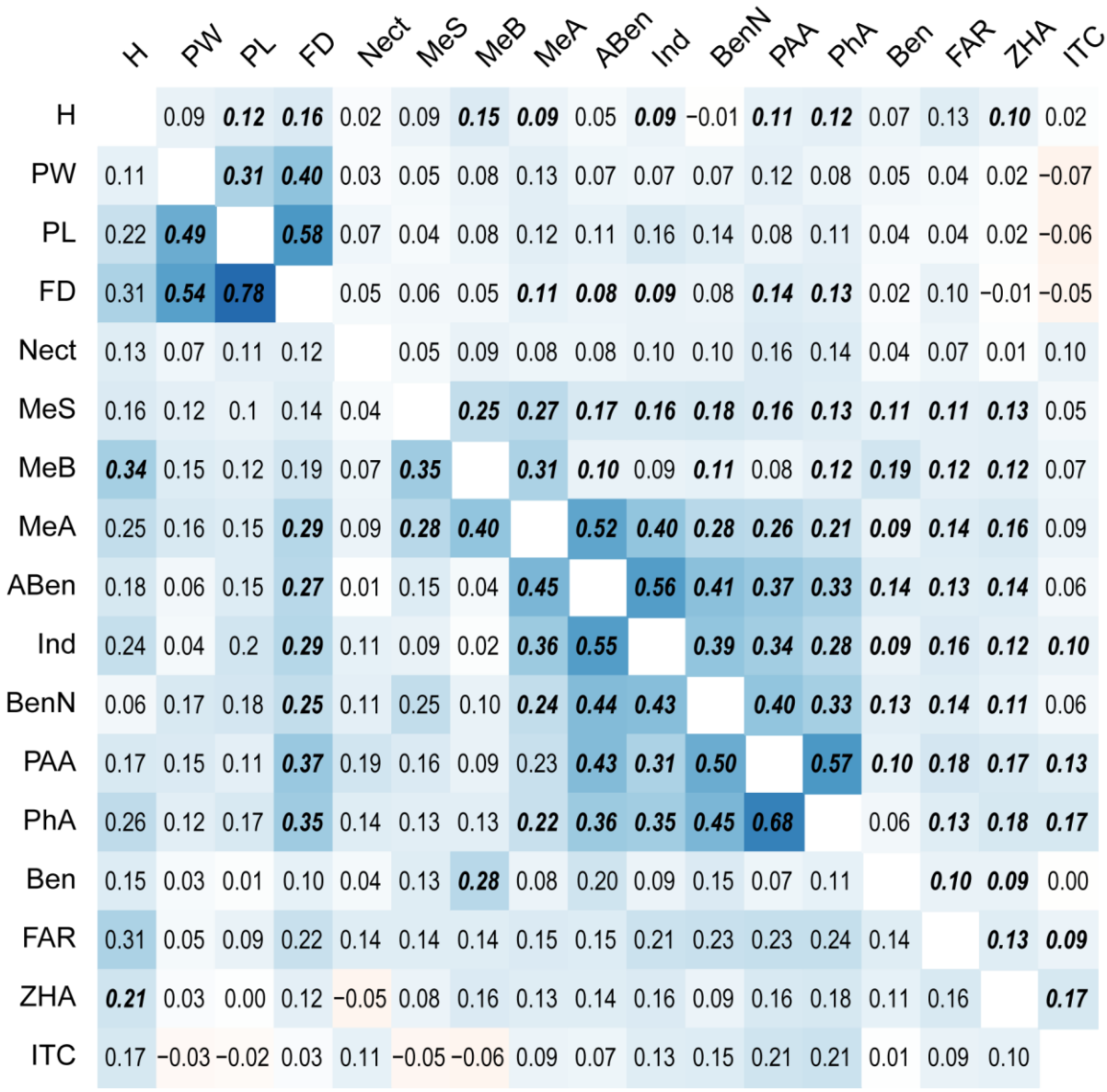
groups ($P < 0.001$). In the linear discriminant analysis (2f), discriminant functions 1 and 2, were significantly different among the groups ($P < 0.001$).

Figure 3. Correlation plot for phenotypic (upper diagonal) and genetic (lower diagonal) correlations of 17 floral traits in *Brassica rapa*. The intensity of color is in proportion to the magnitude of correlations. Correlation coefficients are given in numbers and significant values are in bold italic. Abbreviations of trait names see in Table 1.

Figure 4. Pollinator preference of plants diverging in height in dual-choice, floral constancy bioassay and field experiments. The graph shows percentage landings of pollinators on tall and short line plants from F3 generation. * $P < 0.05$; ns, not significant; numbers in brackets indicate the number of landings.







	H	PW	PL	FD	Nect	MeS	MeB	MeA	ABen	Ind	BenN	PAA	PhA	Ben	FAR	ZHA	ITC
H		0.09	***0.12***	***0.16***	0.02	0.09	***0.15***	***0.09***	0.05	***0.09***	−0.01	***0.11***	***0.12***	0.07	0.13	***0.10***	0.02
PW	0.11		***0.31***	***0.40***	0.03	0.05	0.08	0.13	0.07	0.07	0.07	0.12	0.08	0.05	0.04	0.02	−0.07
PL	0.22	***0.49***		***0.58***	0.07	0.04	0.08	0.12	0.11	0.16	0.14	0.08	0.11	0.04	0.04	0.02	−0.06
FD	0.31	***0.54***	***0.78***		0.05	0.06	0.05	***0.11***	***0.08***	***0.09***	0.08	***0.14***	***0.13***	0.02	0.10	−0.01	−0.05
Nect	0.13	0.07	0.11	0.12		0.05	0.09	0.08	0.08	0.10	0.10	0.16	0.14	0.04	0.07	0.01	0.10
MeS	0.16	0.12	0.1	0.14	0.04		***0.25***	***0.27***	***0.17***	***0.16***	***0.18***	***0.16***	***0.13***	***0.11***	***0.11***	***0.13***	0.05
MeB	***0.34***	0.15	0.12	0.19	0.07	***0.35***		***0.31***	***0.10***	0.09	***0.11***	0.08	***0.12***	***0.19***	***0.12***	***0.12***	0.07
MeA	0.25	0.16	0.15	***0.29***	0.09	***0.28***	***0.40***		***0.52***	***0.40***	***0.28***	***0.26***	***0.21***	***0.09***	***0.14***	***0.16***	0.09
ABen	0.18	0.06	0.15	***0.27***	0.01	0.15	0.04	***0.45***		***0.56***	***0.41***	***0.37***	***0.33***	***0.14***	***0.13***	***0.14***	0.06
Ind	0.24	0.04	0.2	***0.29***	0.11	0.09	0.02	***0.36***	***0.55***		***0.39***	***0.34***	***0.28***	***0.09***	***0.16***	***0.12***	***0.10***
BenN	0.06	0.17	0.18	***0.25***	0.11	0.25	0.10	***0.24***	***0.44***	***0.43***		***0.40***	***0.33***	***0.13***	***0.14***	***0.11***	0.06
PAA	0.17	0.15	0.11	***0.37***	0.19	0.16	0.09	0.23	***0.43***	***0.31***	***0.50***		***0.57***	***0.10***	***0.18***	***0.17***	***0.13***
PhA	0.26	0.12	0.17	***0.35***	0.14	0.13	0.13	***0.22***	***0.36***	***0.35***	***0.45***	***0.68***		0.06	***0.13***	***0.18***	***0.17***
Ben	0.15	0.03	0.01	0.10	0.04	0.13	***0.28***	0.08	0.20	0.09	0.15	0.07	0.11		***0.10***	***0.09***	0.00
FAR	0.31	0.05	0.09	0.22	0.14	0.14	0.14	0.15	0.15	0.21	0.23	0.23	0.24	0.14		***0.13***	***0.09***
ZHA	***0.21***	0.03	0.00	0.12	−0.05	0.08	0.16	0.13	0.14	0.16	0.09	0.16	0.18	0.11	0.16		***0.17***
ITC	0.17	−0.03	−0.02	0.03	0.11	−0.05	−0.06	0.09	0.07	0.13	0.15	0.21	0.21	0.01	0.09	0.10